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Enhanced Bystander Cytotoxicity of P450 Gene-directed Enzyme Prodrug Therapy by Expression of the Antiapoptotic Factor p35[1]

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ABSTRACT

Cytochrome P450 gene-directed enzyme prodrug therapy substantially augments intratumoral activation of anticancer prodrugs, such as cyclophosphamide (CPA), leading to a strong increase in antitumor effect without a corresponding increase in host toxicity. Attempts to additionally increase tumor cell kill by enhancing the intrinsic chemosensitivity of P450-expressing tumor cells by chemical means (depletion of cellular glutathione) or by coexpression of proapoptotic factors was shown to result in the desired increase in chemosensitivity, but with a decrease in net production of bystander cytotoxic drug metabolites because of accelerated death of the prodrug-activating tumor cells. Moreover, tumor cell P450 activity declined during the course of apoptosis induced by P450-activated CPA, limiting the potential of the tumor cell for continued production of activated drug metabolites. This limitation could be overcome by retroviral delivery of the baculovirus-encoded caspase inhibitor p35 to P450-expressing tumor cells. p35 substantially prolonged the activation of CPA by P450 "factory cells," leading to an increase in their bystander cytotoxicity toward P450-deficient tumor cells. This effect was greatest in tumor cells treated with CPA for an 8-h period, a schedule designed to model the effective time period of drug exposure in bolus CPA-treated patients *in vivo*. Notably, retroviral transduction of tumor cells with p35 did not induce drug resistance, as shown by the absence of long-term tumor cell survival or detectable colony formation activity after CPA treatment. These findings demonstrate that antiapoptotic factors, such as p35, can be used in a novel manner to enhance prodrug activation gene therapy by delaying tumor cell death, thereby increasing the net production of bystander cytotoxic metabolites and, hence, the overall effectiveness of the anticancer strategy.

INTRODUCTION

The anticancer prodrug CPA[3] is widely used in the treatment of human cancers. CPA is activated in the liver by a P450-catalyzed hydroxylation reaction that yields 4-OH-CPA, the primary activated metabolite. 4-OH-CPA and its ring-opened derivative, aldophosphamide, are transported via the bloodstream to both tumor and healthy tissues, where they decompose to yield phosphoramide mustard and acrolein, which alkylate DNA and protein, respectively (1). Cell culture and *in vivo* tumor xenograft model studies have demonstrated that a striking increase in tumor cell kill can be achieved without an increase in host toxicity when P450-catalyzed prodrug activation occurs within the target tumor cell. Tumor cells infected with a viral vector encoding P450 2B6, a human liver P450 enzyme that activates CPA with high efficiency (2), are greatly sensitized to the cytotoxic effects of CPA (3), demonstrating the potential utility of mammalian P450 genes for GDEPT (4–7). An important feature of P450 GDEPT is the bystander effect, which provides a mechanism for extending the cytotoxic response beyond the minority of tumor cells transduced with a prodrug-activating P450 gene to encompass surrounding tumor cells, thereby amplifying the cytotoxic effects of the prodrug. Studies of P450-expressing tumor cells cocultured with P450-deficient bystander cells in the form of a monolayer (3, 5) or as spheroids (8) have demonstrated that bystander cytotoxicity is mediated by the release of soluble, activated CPA metabolites, which diffuse freely across cell membranes, circumventing the need for direct cell-to-cell contact to effect bystander cell death.

DNA damage induced by cancer chemotherapeutic drugs, including P450-activated CPA (9), typically leads to tumor cell death by an apoptotic mechanism. Apoptosis is triggered by a complex cascade of events, central to which is the activation of caspases, cysteine proteases that include both upstream (initiator) and downstream (effector) caspases (10). Two major pathways of caspase-dependent apoptosis have been identified. One pathway is initiated by the formation of a cell death-inducing plasma membrane receptor-signaling complex (11), which induces aggregation and activation of the initiator caspase 8 (12–14). A second apoptotic pathway is triggered by cellular stress such as DNA damage (12, 15) and is primarily associated with cell death linked to the release of proapoptotic molecules from the mitochondria (mitochondrial transition) and the subsequent activation of a distinct initiator caspase, caspase 9 (16–18). This mitochondrial-mediated caspase 9 pathway is the key regulatory pathway responsible for CPA-induced tumor cell death (9). Once activated, caspase 8 and caspase 9 cleave, and thereby activate downstream caspases, such as caspases 3 and 7 (19). These downstream effector caspases, in turn, cleave multiple cellular proteins, triggering the phenotypic changes that are associated with apoptosis.

Tumor cell kill can be stimulated by expression of proapoptotic factors, such as Bax, p53, Trail, and various caspases, either alone or in combination with traditional chemotherapy, as shown in both preclinical and clinical anticancer gene therapies (20–24). This approach is based on the observation that proapoptotic factors augment the cytotoxic effects of many cancer chemotherapeutic drugs by lowering the threshold drug concentration required to induce tumor cell death. In the case of CPA and certain other alkylating agents, enhanced cytotoxicity may be achieved by other approaches, including depletion of protective small molecules, such as GSH, or by decreasing the expression or activity of cell-protective enzymes, such as GSH *S*-transferases (25–27) or aldehyde dehydrogenases (28–30), both of which can detoxify activated CPA.

IAPs suppress apoptosis by binding directly to caspases and inhibiting their proteolytic activity. IAPs, first discovered in baculovirus, have been identified in both mammals and lower eukaryotes (31). IAPs can inhibit apoptosis induced by a variety of cell death stimuli, including receptor-dependent death signals such as tumor necrosis factor α and Fas, chemotherapeutic drugs such as etoposide and Taxol, and cellular stress resulting from growth factor withdrawal (31). IAPs do not interfere with the release of cytochrome C mediated by the proapoptotic factor Bax, indicating that IAPs block caspase activation and apoptosis downstream of the mitochondrial transition (32). IAPs such as Survivin are widely expressed in tumor cells (33). Consequently, cancer gene therapeutic strategies have been intro-

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[3] The abbreviations used are: CPA, cyclophosphamide; P450, cytochrome P450; 4-OH-CPA, 4-hydroxy-cyclophosphamide; GDEPT, gene-directed enzyme prodrug therapy; IAP, inhibitor of apoptosis; 9L/P450 cells, 9L gliosarcoma cells that stably expression P450 2B6 and P450 reductase; BSO, buthionine sulfoximine; GSH, glutathione; RT-PCR, reverse transcription-PCR; AIF, apoptosis-inducing factor.

duced with the goal of inhibiting expression of Survivin and other IAPs, and may have potential for clinical development (34).

In the present study, we investigate ways to increase the therapeutic effectiveness of P450 GDEPT by modulating apoptosis in a manner that increases the bystander cytotoxicity associated with tumor cell activation of CPA. We investigate the sensitization of P450-expressing tumor cells to activated CPA by depletion of GSH. We also examine the impact that IAPs have on the intrinsic chemosensitivity of P450-expressing tumor cells and on the ability of the cells to sustain production of activated CPA metabolites. Our findings demonstrate that the baculovirus protein p35 (35), an IAP and potent caspase inhibitor, is able to substantially delay but ultimately not block the cytotoxicity of CPA to P450-expressing tumor cells. This delay, in turn, prolongs the production and release of 4-OH-CPA into the tumor milieu, with a corresponding increase in bystander cytotoxicity toward P450-deficient tumor cells. These findings are discussed in the context of the introduction of IAPs as enhancement factors for P450 and other GDEPT strategies for cancer treatment.

MATERIALS AND METHODS

Generation of Stable Cell Lines. Rat 9L/P450 gliosarcoma cells and derivatives, all prepared by retroviral infection, were grown in DMEM containing 10% fetal bovine serum and cultured as monolayers at 37°C in a humidified atmosphere containing 5% CO_2. 9L/P450 cells, generously provided by Dr. Youssef Jounaidi of this laboratory, coexpress human P450 2B6 and human P450 reductase, and correspond to the 9L/2B6/Red cell line described previously (36). 9L/P450 cells expressing IAPs (*i.e.*, p35, hIAP1, hIAP2, or Survivin) were prepared by retroviral infection, as follows. p35 cDNA (35), obtained from Dr. Thomas D. Gilmore (Boston University, Boston, MA), was subcloned by blunt end ligation from pBluescript/p35 into the *Hind*III and *Cla*I sites of the retroviral plasmid pLNCX (Clontech Laboratories, Palo Alto, CA). hIAP1 and hIAP2 cDNAs, both myc-tagged and cloned into pcDNA3 (Ref. 32; Dr. Eric LaCasse, Apoptogen Inc., Ottawa, Ontario, Canada), and Survivin cDNA in pcDNA3 (33), obtained from Dr. Dario C. Altieri (Yale University, New Haven, CT), were subcloned by directional ligation into the *Hind*III and *Cla*I sites of pLNCX. The generation of retroviral particles was described previously (9). Briefly, the packaging cell line Bosc 23 (37) was transfected with pLNCX vectors containing each of the IAP cDNAs. Medium containing retroviral particles was collected 48 h later and placed on 9L/P450 cells (38). The cells were incubated for 48 h and subsequently selected with 1.5 mg/ml G418 for 5 days. The resultant pools of G418-resistant cells stably expressed the IAP, as shown by RT-PCR. To obtain clonal 9L/P450/p35 cell lines, 12 individual p35-expressing colonies were isolated from the original pool of retrovirally infected cells by plating the cells at a calculated density of 1 cell/well in a 96-well plate. Individual clones were assayed for 4-OH-CPA production and caspase 3 activity after 1 mM CPA treatment. Four colonies that showed a substantial decrease in caspase 3 activity after CPA treatment and yet retained CPA 4-hydroxylase activity were chosen for more detailed analysis. P450 2B6 expression was evaluated by Western blotting (see below), and P450 reductase activity was assayed by cytochrome C reduction (both assayed in whole cell extracts; Ref. 3). p35 expression was confirmed by RT-PCR (see below).

RT-PCR. RNA was isolated from 9L/P450 cells expressing p35 or Survivin using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD). One ml of Trizol was added to each 35-mm dish seeded with 2×10^5 cells. The cell lysate was passed several times through a pipette tip, incubated for 5 min, and transferred to a clean tube. Chloroform (200 μl) was added to each tube, and the samples were mixed, incubated for 3 min, and centrifuged at 11,000 $\times$ *g* for 15 min at 4°C. RNA was precipitated from the aqueous phase by incubation with 0.5 ml of isopropyl alcohol for 10 min followed by centrifugation at 11,000 $\times$ *g* for 10 min at 4°C. The RNA pellet was washed once with cold 75% ethanol in diethyl pyrocarbonate-treated water and centrifuged at 7,000 $\times$ *g* for 5 min at 4°C. The pellet was resuspended in 50 μl of diethyl pyrocarbonate-treated water and incubated for 10 min at 60°C. First-strand synthesis was performed using the SuperScript preamplification system (Life Technologies, Inc.). Total RNA (2 μg) was mixed with 0.5 μg of oligo$(dT)_{12-18}$ and then incubated at 70°C for 10 min. The reaction mix was cooled to room temperature followed by addition of 50 μl of 10$\times$ PCR buffer, 25 mM $MgCl_2$, 10 mM deoxynucleotide triphosphate mix, and 0.1 M DTT. The total mix was incubated at 42°C for 5 min. SuperScript II reverse transcriptase (200 units) was then added to each tube and incubated for an additional 50 min. The reaction was terminated by incubation at 70°C for 15 min. Samples were then incubated for 20 min at 37°C with RNase H (USB, Cleveland, OH). PCR was performed using 5 μl of the RNase H-digested first-strand synthesis product in reactions containing 10 μM gene-specific primers (for p35 or Survivin). Samples were incubated at 94°C for 3 min, then subjected to 35 PCR cycles (94°C for 1 min, 56°C for 1 min, and 72°C for 1 min). The expected PCR product lengths were 561 bp (p35-specific primers: 5′-cctcccgctgttttgacctccta-3′ and 5′-atcccggcttcaacacgcatacc-3′) and 614 bp (Survivin-specific primers: 5′-gtgggccccttagcaatgtcttag-3′ and 5′-caccccgtttc-cccaatga-3′).

Western Blotting. 9L/P450 cell extracts (20 μg) prepared in lysis buffer [20% glycerol, 1% Triton X-100, 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM DTT, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mg/ml pepstatin] were analyzed by Western blotting (9). P450 2B6 protein was detected using monoclonal anti-P450 2B6 antibody at a dilution of 1:5000 (Gentest Corp., Woburn, MA). P450 reductase was detected with an antipeptide antibody (1:5000 dilution) obtained from Dr. R. Edwards (Royal Postgraduate Medical School, London, United Kingdom). After washing, the blots were incubated with goat antimouse or goat antirabbit HRP-linked secondary antibody (Pierce, Rockford, IL). Blots were visualized with enhanced chemiluminescence Western blotting detection reagent (Amersham Pharmacia Biotech) and exposed to Kodak X-OMAT blue film XB-1. Western blot films were scanned with ScanWizard v5.02 software and saved as TIFF files. Densitometric values were obtained using IQMac v1.2 software.

Growth Inhibition Assay. 9L/P450 and 9L/P450-derived cells lines were routinely plated at 1.5×10^5 cells/well in 12-well dishes containing 1.5 ml medium/well. Cells were grown overnight and then treated, either continuously or for 8 h, with culture medium containing 1 mM CPA, as specified in each experiment, with 0 h corresponding to the time of CPA addition, unless indicated otherwise. Cells remaining on the plate at the indicated times were washed twice with cold PBS and then stained with crystal violet solution (9). The stain was eluted from the cells and the absorbance read at 595 nm to determine the relative protein content of each sample (relative cell number; Ref. 3).

Caspase Activity Measurements. Caspase activities were assayed as described previously (9). Briefly, 9L/P450 and 9L/P450-derived cells were treated with CPA for the times indicated in each experiment. Floating and attached cells were collected, pooled, dissolved in lysis buffer {10 mM HEPES buffer (pH 7.4) containing 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1 -propanesulfonic acid detergent, 5 mM DTT, 350 ng/ml phenylmethylsulfonyl fluoride, 10 ng/ml pepstatin A, 10 ng/ml aprotinin, and 20 ng/ml leupeptin} and then subjected to three freeze-thaw cycles. Lysates were spun, and the supernatant (cell extract) was assayed for caspase 3, 8, and 9 activities by incubation with caspase form-selective substrates: Ac-DEVD-AMC (Biomol) for caspase 3 (39, 40), LETD-AFC (Bio-Rad) for caspase 8, and Ac-LEHD-AFC (Bio-Rad) for caspase 9. Assays were carried out at 37°C for 15 min (caspase 3) or 3 h (caspases 8 and 9). Background activity was determined for each sample by preincubation with or without caspase form-selective inhibitor (9).

Quantitation of Cellular 4-OH-CPA Production. 9L/P450 cells and 9L/P450-derived cell lines were plated in 12-well tissue culture plates at 1.5×10^5 cells/well with 1.5 ml medium/well. Cellular CPA 4-hydroxylase activity was determined at the times indicated in each figure by incubation for a 4-h period with fresh medium containing 1 mM CPA. Five mM semicarbazide was included in the medium to trap and stabilize the initial 4-OH-CPA metabolite. An aliquot of medium (0.5 ml) was removed from each well, and snap frozen in liquid nitrogen and stored at −80°C until ready for processing and 4-OH-CPA analysis. Cells remaining on the plate were washed with 1$\times$ PBS and stained with crystal violet (A_{595}). Protein in the 0.5-ml culture medium sample was precipitated by the sequential addition of 200 μl of ice-cold $ZnSO_4$, 200 μl of saturated barium hydroxide, and 100 μl of 0.01 M HCl. Samples were spun at 14,000 rpm for 20 min, and 750 μl of the supernatant was transferred to a new tube. Acrolein was derivatized with 3-aminophenol by the addition of 400 μl of fluorescent reagent (6 mg of 3-aminophenol mixed with 6 mg of

hydroxylamine hydrochloride in 1 ml of 1 N HCl). The samples were heated at 90°C for 20 min in the dark, cooled to room temperature, and analyzed (100 μl) on a C18 high-performance liquid chromatography column (41). Calibration curves were generated using 4-OOH-CPA dissolved in cell culture medium. Cellular CPA 4-hydroxylase-specific activity was then calculated (nmol 4-OH-CPA/ml media/A_{595}).

Bystander Experiments. Two bystander experiment protocols were used. In protocol 1 (direct bystander cell contact protocol), 9L/LacZ cells (bystander cells) were cocultured with either 9L/P450 or 9L/P450/p35–9 cells. In protocol 2 (medium contact bystander protocol), the bystander cells were separated from the P450-expressing 9L cells by a permeable membrane. For protocol 1, 9L/P450 and 9L/P450/p35–9 cells were plated in triplicate at 0.5×10^5 and 0.75×10^5 cells/well, respectively, in 12-well tissue culture dishes containing 1.5 ml of culture medium/well. The higher number of 9L/P450/p35–9 cells compensates for the lower intrinsic CPA 4-hydroxylase activity of this cell line compared with 9L/P450 cells (see Figs. 4 and 5, below). Twenty-four h after seeding, the cells were treated with 1 mM CPA for 8 h. Forty-eight h after beginning the first drug treatment, 9L/LacZ bystander cells were plated on top of the 9L/P450 and 9L/P450/p35–9 cell monolayers at 2.5×10^5 9L/LacZ cells/well. Twenty-four h later the mixed cell populations were treated with 0, 0.125, 0.25, or 0.5 mM CPA for a second 8-h drug treatment period. The cells were then grown in drug-free medium for an additional 16 h (*i.e.*, 24 h from the start of the second drug treatment), at which point the cells were trypsinized, collected, counted, and replated at various densities in six-well plates to allow for the growth of individual colonies, as follows: drug-free control cells were replated in duplicate at 100, 150, and 250 cells/well. CPA-treated 9L/P450 + 9L/LacZ cells were replated as follows: 500, 1,000, and 2,000 cells/well (0.125 mM CPA-treated samples); 1,000, 2,000, and 4,000 cells/well (0.25 mM CPA-treated samples); and 2,000, 5,000, and 10,000 cells/well (0.5 mM CPA-treated samples). CPA-treated 9L/P450/p35–9 + 9L/LacZ cells were replated at twice the densities shown above. Cells were grown for 10–11 days, with medium changes every 3 days, and then stained with X-Gal. lacZ-positive colonies containing ≥50 cells were counted. Cells were then restained with crystal violet, and the total number of colonies was determined. The number of 9L/P450 or 9L/P450/p35–9 colonies on each plate was then calculated by subtracting number of the lacZ-positive colonies from each sample. The colony efficiency (number of colonies per number of cells seeded) was then calculated for each treatment group.

For protocol 2, 9L/P450 and 9L/P450/p35–9 cells were plated at 0.6×10^5 cells and 0.9×10^5 cells/well, respectively, on cell culture inserts (0.4 μM PET Track-etched membrane 3090; B-D Labware, Franklin Lakes, NJ). The inserts were suspended in six-well tissue culture dishes containing 3 ml of medium/well. Twenty-four h after plating, the cells were treated with 1 mM of CPA for 8 h. Forty-eight h after beginning the first drug treatment, 9L/LacZ cells were plated in a separate set of six-well tissue dishes at 6×10^5 cells/well. Four h later the inserts containing 9L/P450 or 9L/P450/p35–9 cells were placed into the 9L/LacZ-seeded wells. Twenty-four h after plating the 9L/LacZ cells, the wells (containing 9L/LacZ +9L/P450 or 9L/LacZ +9L/P450/p35–9 cells) were treated with 0, 0.125, 0.25, or 0.5 mM CPA for an 8-h period. The drug-treated cells were then grown in drug-free medium for 16 h, at which point the two populations of cells in each well [*i.e.*, 9L/Lac Z (lower chamber) and 9L/P450 (or 9L/P450/p35–9) cells (upper chamber)] were trypsinized, collected separately, counted, and replated at various densities in six-well dishes for a colony formation assay as described for protocol 1, but at the following cell densities: drug-free control cells were replated in duplicate at 100, 150, and 250 cells/well; and 9L/LacZ +9L/P450 samples at 250, 500, and 1000 cells/well (0.125 mM CPA), 500, 1,000, and 2,000 cells/well (0.25 mM CPA), or 1,000, 2,000 and 5,000 cells/well (0.5 mM CPA). The 9L/LacZ +9L/P450/p35–9 samples were replated at 2,000, 4,000, and 7,000 cells/well (0.125 mM CPA), 5,000, 10,000 and 20,000 cells/well (0.25 mM CPA), and 10,000, 20,000 and 40,000 cells/well (0.5 mM CPA). Cells were grown for 10–11 days, stained with crystal violet, and colony formation then determined.

RESULTS

GSH Depletion Sensitizes 9L/P450 Cells to CPA but Decreases Net Production of 4-OH-CPA. P450 2B6 substantially enhances the chemosensitivity of tumor cells to P450-activated anticancer prodrugs, such as CPA. Tumor cells can also be sensitized to activated CPA using the GSH synthesis inhibitor BSO, which depletes intracellular GSH and thereby decreases cellular GSH *S*-transferase activity, which has been associated with inactivation of and resistance to 4-OH-CPA and other anticancer alkylating agents (26, 42, 43). Therefore, we anticipated that the depletion of GSH in P450-expressing tumor cells by BSO pretreatment (50 μM for 24 h) would: (*a*) suppress the inactivation of 4-OH-CPA by cellular GSH, thereby increasing intracellular levels of 4-OH-CPA leading to increased 4-OH-CPA released into the culture medium; and (*b*) enhance the cytotoxicity of CPA toward the P450-expressing tumor cells and toward P450-deficient bystander tumor cells. To test these hypotheses, 9L tumor cells infected with retrovirus encoding P450 2B6 were pretreated with BSO and then exposed to CPA. BSO treatment alone had no effect on cell survival; however, it substantially increased the sensitivity of the cells to CPA, as indicated by visual examination of the culture for membrane blebbing (data not shown), which is an indicator of apoptosis, and by the effects of BSO on cell survival, which was decreased from 62% to 13% at 72 h (Fig. 1*A*). However, analysis of the CPA 4-hydroxylase (prodrug activation) activity of the 9L/P450 cells revealed an 86% decrease in the capacity of the BSO-pretreated cell to produce and release 4-OH-CPA into the culture medium by 24 h of CPA treatment as compared with a 53% decrease in the absence of BSO (Fig. 1*B*, *left*). These decreases in cellular CPA 4-hydroxylase

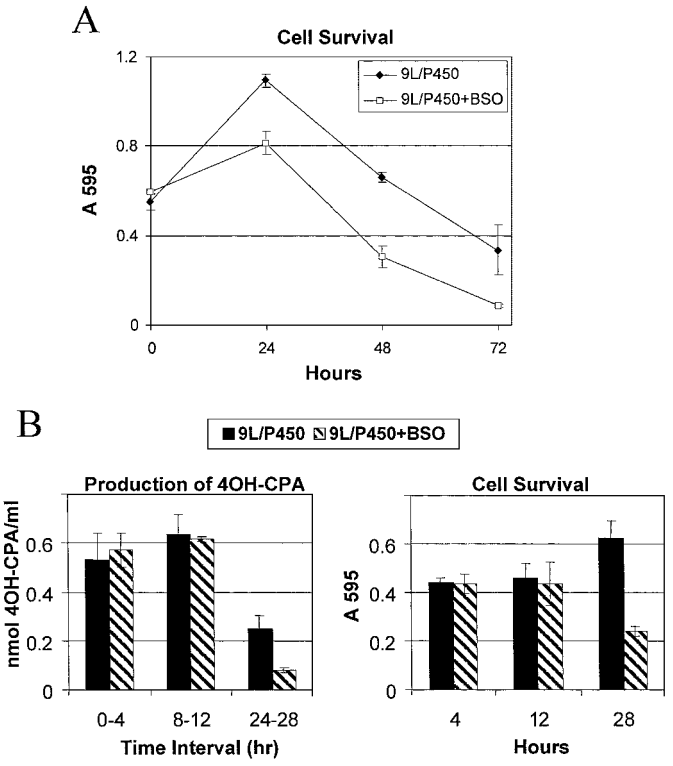

Fig. 1. GSH depletion enhances CPA-induced cytotoxicity and decreases CPA 4-hydroxylase activity in 9L/P450 cells. *A*, 9L/P450 cells were either untreated or were pretreated with 50 μM BSO for 24 h. Cells were then treated with 1 mM CPA, beginning at time 0, for times up to 72 h, as indicated. Duplicate samples were stained with crystal violet (A_{595}) at each time point to quantitate relative cell protein content (*cell survival*). Mean values (± range) were graphed. The 24-h BSO pretreatment had no effect on cell growth, as seen by the coincidence of A_{595} values at time 0. *B*, 9L/P450 cells pretreated with BSO, as in *A*, were treated with 1 mM CPA for times up to 28 h. Cellular CPA 4-hydroxylase activity was assayed by incubating the cells in fresh medium containing 1 mM CPA and 5 mM semicarbazide, added to stabilize the 4-OH-CPA metabolite, for the 4-h time interval indicated at the *bottom of B, left side*. An aliquot (0.5 ml) of medium was removed, derivatized, and then analyzed for 4-OH-CPA as described in "Materials and Methods." Shown are the measured culture medium concentrations of 4-OH-CPA (mean ± range for $n = 2$; *left*). Shown on the *right* are crystal violet values, indicative of relative cell protein content of each sample; *bars*, ±SD.

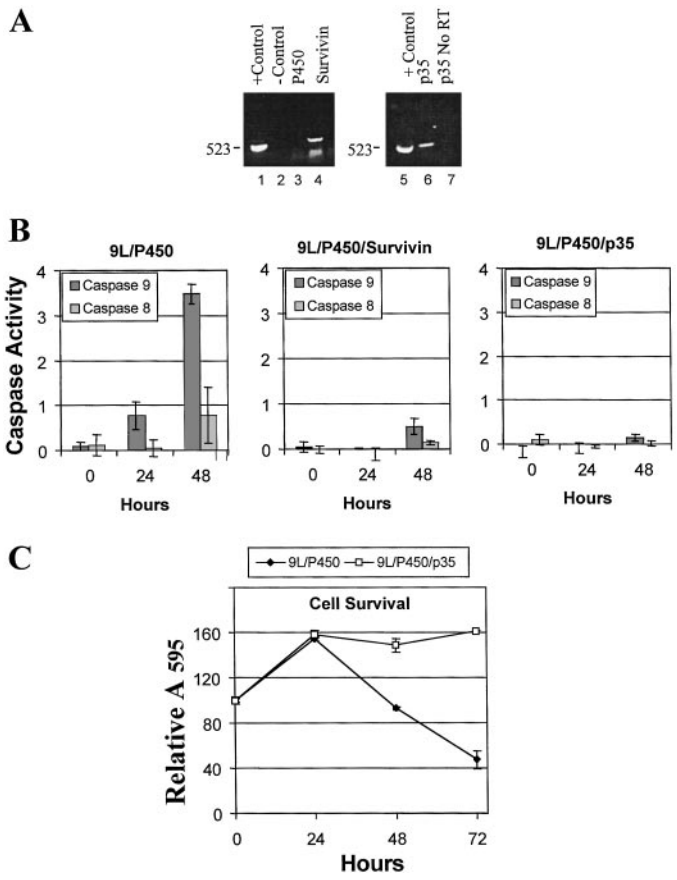

Fig. 2. IAP expression in 9L/P450 cells. 9L/P450 cells were infected with retrovirus encoding the IAPs p35 or Survivin. IAP expression was verified by RT-PCR (*A*). 9L/P450 and 9L/P450-derived IAP cell lines were treated with 1 mM CPA and assayed for caspase 8 or caspase 9 activity as described in "Materials and Methods." Shown in *B* are the caspase 8 and caspase 9 activities of the individual cell lines (mean ± half the range for $n = 2$ separate experiments). Additionally, 9L/P450 and 9L/P450/p35 cells were treated with 1 mM CPA for 72 h and stained with crystal violet at each time point to quantitate cell survival (*C;* mean ± half the range, $n = 2$); *bars,* ±SD.

activity were not seen at earlier time points after CPA addition, and in the case of the BSO-pretreated cells, were associated with an increase in CPA cytotoxicity (Fig. 1*B, right*). Thus, whereas BSO increased the cytotoxicity of CPA to P450-expressing tumor cells, it had the undesirable effect of decreasing the net production of activated prodrug released into the culture medium.

IAPs Inhibit Caspase Activation and Protect 9L/P450 Cells from CPA-induced Cell Death. Current gene therapy technologies typically result in the introduction of the gene therapy vector into only a small fraction of the target tumor cell population. We hypothesized that the greatest bystander cytotoxic effect, and hence the greatest overall antitumor effect, would be achieved by imparting transient drug resistance to those tumor cells that are transduced with the gene therapy vector. This would result in a more efficient prodrug activating factory cell, *i.e.,* one that produces larger quantities of activated prodrug metabolites over an extended period of time before it itself succumbs to prodrug-induced cell death. We have shown previously that 9L/P450 cells undergo caspase 9-dependent apoptosis after CPA treatment (9). Therefore, we investigated whether one or more IAPs, which inhibit caspase activity, could be used to prolong the survival of 9L/P450 cells and thereby enable the cells to produce 4-OH-CPA, the activated metabolite, for an extended time period. 9L/P450 cells that express IAPs were prepared using a retroviral protocol that generates a large pool of independent clones, each containing the IAP cDNA at a distinct site of integration (Fig. 2*A*; data not shown). Of the four IAPs investigated (hIAP1, hIAP2, Survivin, and p35), Survivin and p35 were the most effective at inhibiting of caspase activity in the CPA-treated 9L/P450 cells (Fig. 2*B*; data not shown). p35 effected a virtually complete block in CPA-stimulated caspase activity (9L/P450/p35 cells; Fig. 2*B, right*) and was selected for additional investigation. Other studies revealed that the expression of p35 imparted a cytostatic response when 9L/P450 cells were treated with CPA (1 mM for 72 h); this contrasts with the cytotoxic response seen in the absence of p35 (Fig. 2*C*). Expression of the antiapoptotic factor Bcl-2 also changes the cellular response to CPA from a cytotoxic response to a cytostatic response (9).

p35 Sustains the Ability of 9L/P450 Cells to Activate CPA. Initial experiments suggested that the prolonged viability of CPA-treated 9L/P450/p35 cells compared with CPA-treated 9L/P450 cells may help prolong cellular CPA 4-hydroxylase activity. This possibility was indicated by the 53% decrease in the capacity of 9L/P450 cells to activate CPA 24 h after the initial drug exposure, as compared with only a 34% decrease in the rate of CPA activation by 9L/P450/p35 cells under the same conditions (Fig. 3*A, left panel*). The continuous CPA treatment schedule used in this *in vitro* experiment mimics the continuous 4-day CPA infusion protocol used in patients receiving high-dose CPA in combination with autologous bone marrow transplantation (44, 45). However, this schedule differs from conventional CPA treatment protocols where CPA is administered as a bolus, and where the half-lives of CPA and its active phosphoramide mustard metabolite are both ~8 h in adult cancer patients (46, 47). To more closely mimic this latter CPA treatment schedule, 9L/P450 cells were treated with CPA for 8 h, and then assayed for their ability to survive and to metabolize CPA to 4-OH-CPA. Twenty-four h after an 8-h exposure to CPA, the p35-deficient 9L/P450 cells retained 97% of their initial CPA 4-hydroxylase activity. However, by 72 h, CPA metabolic activity was reduced to only ~30% of the initial activity level (Fig. 3*B, left panel*). By contrast, 9L/P450/p35 cells displayed an increase in P450 activity over the course of the experiment, with a ~6-fold higher CPA 4-hydroxylase activity compared with 9L/P450 cells achieved at the 72-h time point. This differential effect of 8 h of CPA treatment on 9L/P450 *versus* 9L/P450/p35 cells reflects two factors: (*a*) a decrease in the survival of 9L/P450 cells but not 9L/P450/p35 cells (Fig. 3*B, middle panel*); and (*b*) a more rapid, and more complete, loss of CPA 4-hydroxylase activity in the CPA-treated 9L/P450 cells (Fig. 3*B, left panel*). Overall, the p35-expressing cells exhibited approximately a 2–3-fold higher CPA 4-hydroxylase specific activity [calculated by normalizing 4-OH-CPA production to total cell staining (A_{595})] beginning 24 h after the initial 8-h drug treatment period (Fig. 3*B, right pan*el). This differential in CPA 4-hydroxylase-specific activity between the two cell lines was narrowed substantially when using the continuous CPA treatment protocol (24–28 h time point; Fig. 3*A, right panel*). Of note, the 9L/P450 and 9L/P450/p35 cells displayed the same CPA 4-hydroxylase-specific activity before CPA treatment (0–4 h CPA samples, Fig. 3, *A* and *B*).

Characterization of 9L/P450/p35 Clones Expressing Differing Amount of p35 and P450 Reductase but Similar Amounts of P450. To further investigate the effects of p35 on P450-catalyzed CPA activation, four p35-expressing clones derived from the 9L/P450 cell line were isolated (see "Materials and Methods"). The clones were characterized by RT-PCR as having a level of p35 that was either low (clones 3 and 7; designated p35–3 and p35–7) or high (clones 8 and 9; p35–8 and p35–9; Fig. 4*A*). All four of the clones showed a substantial decrease in CPA-activated caspase 3 activity compared to 9L/P450 cells (Fig. 4*B*). Western blot analysis revealed similar P450 2B6 levels to that of the original 9L/P450 cell line in three of the clones but lower P450 protein levels in clone p35–9 (Fig. 4*C*). By

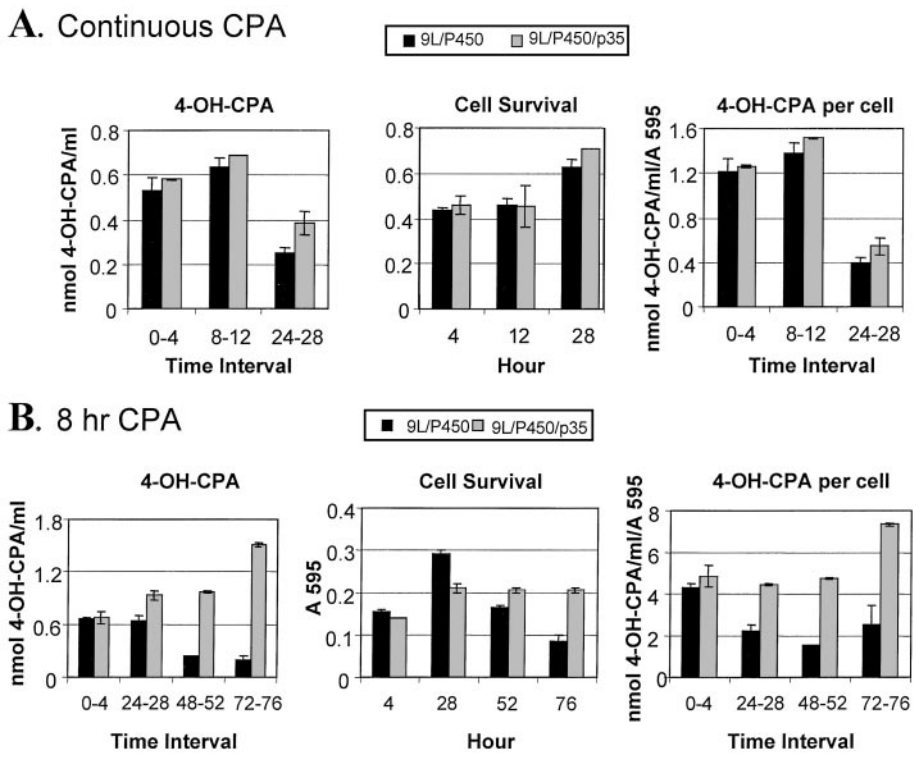

Fig. 3. 9L/P450 cells that express p35 maintain CPA 4-hydroxylase activity when treated with CPA for 8 h but not when treated with CPA continuously. 9L/P450 and 9L/P450/p35 cells were treated with 1 mM CPA continuously (*A*) or were treated with 1 mM CPA for 8 h and then incubated in drug-free medium for the duration of the experiment. Cellular CPA 4-hydroxylase activity at each time point (*left side of each panel*) was assayed as described in Fig. 1. Relative cell protein content (*cell survival*) was determined by crystal violet staining at the time points indicated (A_{595}, *middle*). Cellular CPA 4-hydroxylase activity normalized to total cell protein was calculated by dividing the values shown on the *left panel* by the A_{595} values shown in the *middle panel* (nmol 4-OH-CPA per ml medium per A_{595}; *right*); *bars*, ±SD.

contrast, the P450 reductase activity of the individual clones was variable (Fig. 4*D*). These findings reflect the origin of the 9L/P450 cell line used to prepare the p35 clones: a single clone of P450 2B6-expressing 9L cells was originally infected with a P450 reductase-expressing retrovirus to generate a pool of 9L/P450 cells, comprised of cells containing variable levels of P450 reductase (3, 36). Subsequent infection of this pool of cells with retrovirus-encoding p35 gave rise to the variability in both p35 and P450 reductase expression seen in the individual clones in Fig. 4.

9L/P450 Cells That Express High Levels of p35 Maintain Their Ability to Activate CPA. Fig. 5 depicts CPA 4-hydroxylase activities (Fig. 5, *left*) and relative cellular growth rates (Fig. 5, *center*) for 9L/P450 cells and four 9L/P450/p35 clones. Cells were untreated (Fig. 5*A*, *across*), or were treated with CPA either continuously (Fig. 5*B*) or as a single 8-h exposure (Fig. 5*C*). All five of the cell lines exhibited similar growth rates in the absence of drug treatment (Fig. 5*A*, *center*); however, the intrinsic ability of each line to activate CPA varied (Fig. 5*A*, *left*). 9L/P450 cells and the low p35-expressing clone p35–3 displayed essentially the same rate of 4-OH-CPA formation over a 72-h period, whereas clone p35–7 displayed an increased initial rate of CPA metabolism (t = 0 data; Fig. 5, *A–C*), which may be explained by its elevated P450 reductase activity (compare Fig. 4*D*). Of the two high p35-expressing clones, p35–8 displayed a substantially increased rate of 4-OH-CPA production at all of the time points when compared with 9L/P450 cells (compare elevated P450 reductase activity of this clone; Fig. 4*D*), whereas p35–9 showed a somewhat reduced initial rate of CPA metabolite formation, consistent with its lower P450 2B6 protein content (Fig. 4*C*). Under conditions of continuous CPA treatment (Fig. 5*B*), only clone p35–9 showed a significant increase in cell survival and prolonged formation of 4-OH-CPA (48 and 72 h; Fig. 5*B*, *left*). However, with the 8-h CPA treatment schedule, both clonal lines that express high levels of p35 showed a significant time-dependent increase in 4-OH-CPA production, with a very substantial 6.8–7.8-fold elevation of CPA 4-hydroxylase activity observed at the 72-h time point compared with the p35-deficient 9L/P450 controls (Fig. 5*C*, *left*). This elevated CPA 4-hydroxylase activity in part reflects maintenance by p35 of the cell P450 specific activity, and contrasts with the CPA-induced decrease in P450-specific activity that occurred in the absence of p35 (Fig. 5*C*, *right*). The ability of 9L/P450/p35–8 cells to survive and to maintain high CPA 4-hydroxylase activity for prolonged time is notable, given that these cells generate (and are exposed to) ~2.5-fold higher concentrations of the cytotoxic metabolite 4-OH-CPA during the 8-h drug exposure period than the corresponding p35–9 cells (Fig. 5*C*, *left*).

Coexpression of p35 Enhances the P450-mediated Bystander Effect. We next investigated whether the enhanced 4-hydroxylase activity exhibited by the 9L/P450/p35–9 cells at the time of the second 8-h exposure to CPA would result in an increase in bystander killing by these cells. Whereas 9L/P450 and 9L/P450/p35–9 cells were both able to confer killing on 9L/LacZ bystander cells, as determined in a colony formation assay (white bars, Fig. 6), the extent of bystander killing in response to a second CPA treatment was much greater in the case 9L/P450/p35–9 cells, as seen in a mixed culture experiment (Fig. 6, *B versus A*). Moreover, when the bystander cells were separated from the 9L/P450 cells by a permeable membrane, CPA-pretreated 9L/P450 cells conferred little or no bystander cell killing in response to a second CPA treatment, whereas 9L/P450/p35–9 cells were able to confer 70–80% bystander cell killing under the same conditions (Fig. 6, *D versus C*).

p35 Expression Does Not Block CPA-induced Tumor Cell Death. We next evaluated the impact of high levels of p35 expression (*e.g.*, in p35–9 cells) on the ultimate fate of CPA-treated 9L/P450 cells. Fig. 7 shows that whereas p35 may delay tumor cell death, 9L/P450 tumor cells expressing p35 are eventually killed by CPA. 9L/P450 cells treated with CPA continuously died within 3–4 days, whereas an ~8-day period was required for the death of 9L/P450/p35–9 cells (Fig. 7*B*). By contrast, a single 8-h CPA treatment was not sufficient to kill 9L/P450 or 9L/P450/p35–9 cells. In the case of 9L/P450 cells, a substantial decrease in cell number was seen after 9 days, but this was followed by regrowth of individual colonies by

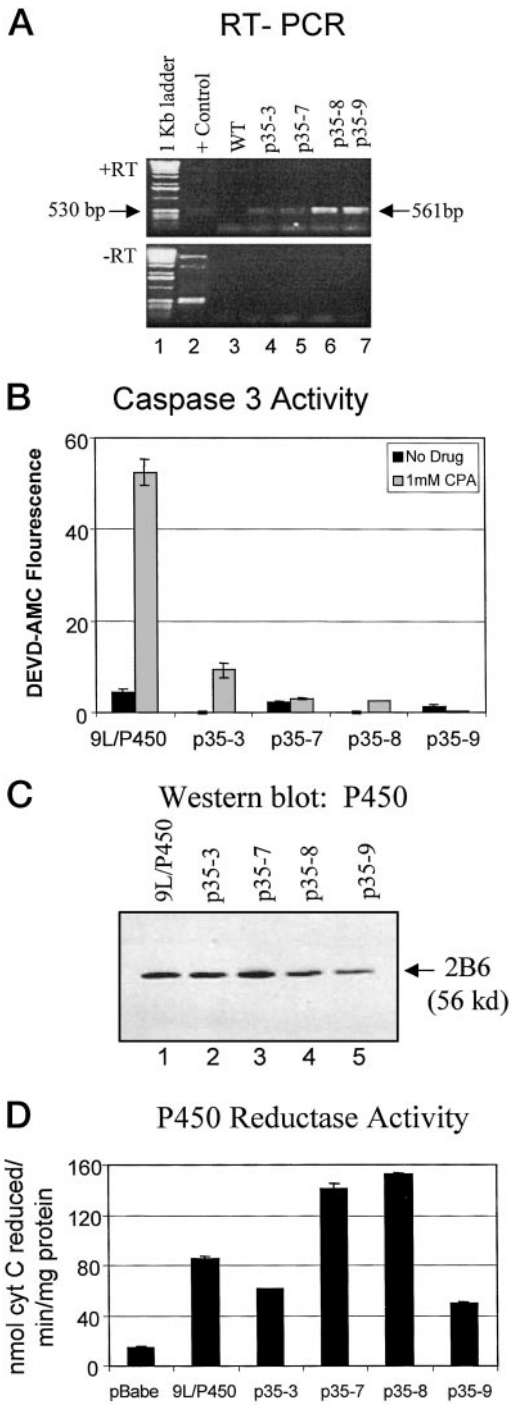

Fig. 4. Characterization of clonal 9L/P450/p35 cell lines. Four clones of 9L/P450/p35 cells were generated as described in "Materials and Methods." Total RNA isolated from the parental 9L/P450 cells (*WT*) and from the four 9L/P450/p35 clonal cell lines was amplified by RT-PCR using p35-specific primers. The 561-bp cDNA product obtained in samples prepared either with (+*RT*) or without (−*RT*) reverse transcriptase was analyzed by agarose gel electrophoresis (*A*). Caspase 3 activity was assayed for each cell line after no drug treatment or treatment with 1 mM CPA for 48 h (mean ± half the range, $n = 2$ separate experiments; *B*). P450 2B6 protein content of the 9L/P450 cells and 9L/P450/p35 clonal cell lines was determined by Western blot analysis (20 μg total cell extract protein/lane; *C*). Total cell extract from each cell line was assayed for P450 reductase activity by monitoring the reduction of cytochrome C (mean ± half the range, $n = 2$; *D*); *bars*, ±SD.

13–17 days (Fig. 7*C*). However, when the cells were given three 8-h CPA treatments (Fig. 7*D*, *vertical arrows*), all of the 9L/P450 cells died by days 6–9 and the 9L/P450/p35–9 cells by day 17. These growth inhibition data reflect a true loss of tumor cell viability, as demonstrated in a colony formation assay. 9L/P450 and 9L/P450/p35–9 cells were treated with CPA for 8 h on day 0 and again on day 3, and then incubated in drug-free medium until day 6, at which time the cells were replated and colony formation quantitated 12 days later. 9L/P450 and 9L/P450/p35–9 cells both displayed similar colony formation activity in the absence of CPA treatment (Table 1). Furthermore, 9L/P450/p35–9 cells showed ~2-fold higher colony formation activity compared with 9L/P450 cells after a single 8-h CPA treatment ($P < 0.02$). This difference reflects the lower initial exposure to 4-OH-CPA of the 9L/P450/p35–9 cells used in this experiment (compare lower intrinsic CPA 4-hydroxylase activity of the p35–9 clone; Fig. 5). However, when two 8-h CPA treatments, spaced 3 days apart, were applied to the cells, no viable colonies were formed by either cell line. Thus, even in 9L/P450/p35–9 cells, which express the highest level of p35 and a reduced level of P450, two 8-h CPA treatments are sufficient to effect complete cell killing, in agreement with the loss of colony formation activity seen for p35 cells in Fig. 6.

Analysis of the CPA 4-hydroxylase activity of the 9L/P450 and 9L/P450/p35–9 cultures revealed comparable levels of 4-OH-CPA production during the initial 8-h drug treatment (Fig. 8, data points plotted on Y axis) and only a modest difference between the two cell lines when they were treated with CPA continuously (Fig. 8*B*, compare 3-day data points). By contrast, a substantial difference in cellular CPA 4-hydroxylase activity was seen at the time of the second CPA treatment (Fig. 8*C*). However, by day 6, *i.e.*, 3 days after the second 8-h CPA treatment, both the 9L/P450 cells and the 9L/P450/p35–9 cells had lost their ability to activate CPA (Fig. 8*C*).

DISCUSSION

An important feature of GDEPT strategies, including P450 GDEPT, is the bystander effect, which can amplify the cytotoxic effect of an activated prodrug and extend it to include tumor cells proximal to those that express the therapeutic gene (6, 48, 49). Activated metabolites of CPA produced by tumor cells infected with a viral vector delivering a P450 2B enzyme kill P450-deficient tumor cells in a cell contact-independent manner. However, the P450-expressing tumor cells are somewhat more sensitive to the activated drug than the P450-deficient tumor cells (5, 50). The present study was undertaken to optimize this bystander cytotoxic potential of P450-expressing tumor cells. Depletion of cellular GSH by BSO treatment was investigated and shown to increase the sensitivity of the tumor cell to activated CPA. However, this treatment also decreased the potential of the tumor cell to produce activated, bystander-cytotoxic CPA metabolites (Fig. 1). A decrease in bystander cytotoxic potential was also observed when P450-expressing tumor cells were treated with CPA in a continuous manner. Continuous CPA-treated tumor cells were shown to lose functional P450 activity relatively early during the course of drug-induced cell death, thereby shortening the time frame during which the cells generate and release activated CPA metabolites into the surrounding medium (Fig. 1*B*). When CPA treatment was limited to an 8-h exposure period, the cells died more slowly; however, their ability to continue to activate CPA did not improve greatly. By contrast, when retrovirus encoding the antiapoptotic factor and caspase inhibitor p35 was used to infect the P450-expressing tumor cells, the potential of the tumor cells for continued production of active CPA metabolites was greatly enhanced.

Retroviral expression of p35 in combination with the CPA-activating P450 2B6 had three important effects: (*a*) it prolonged tumor cell longevity after bolus (8 h) CPA treatment; (*b*) it maintained the capacity of the tumor cell to activate CPA for a prolonged time period; and (*c*) it substantially increased P450-mediated bystander killing. Overall, p35 increased up to ~5-fold the CPA 4-hydroxylase-specific

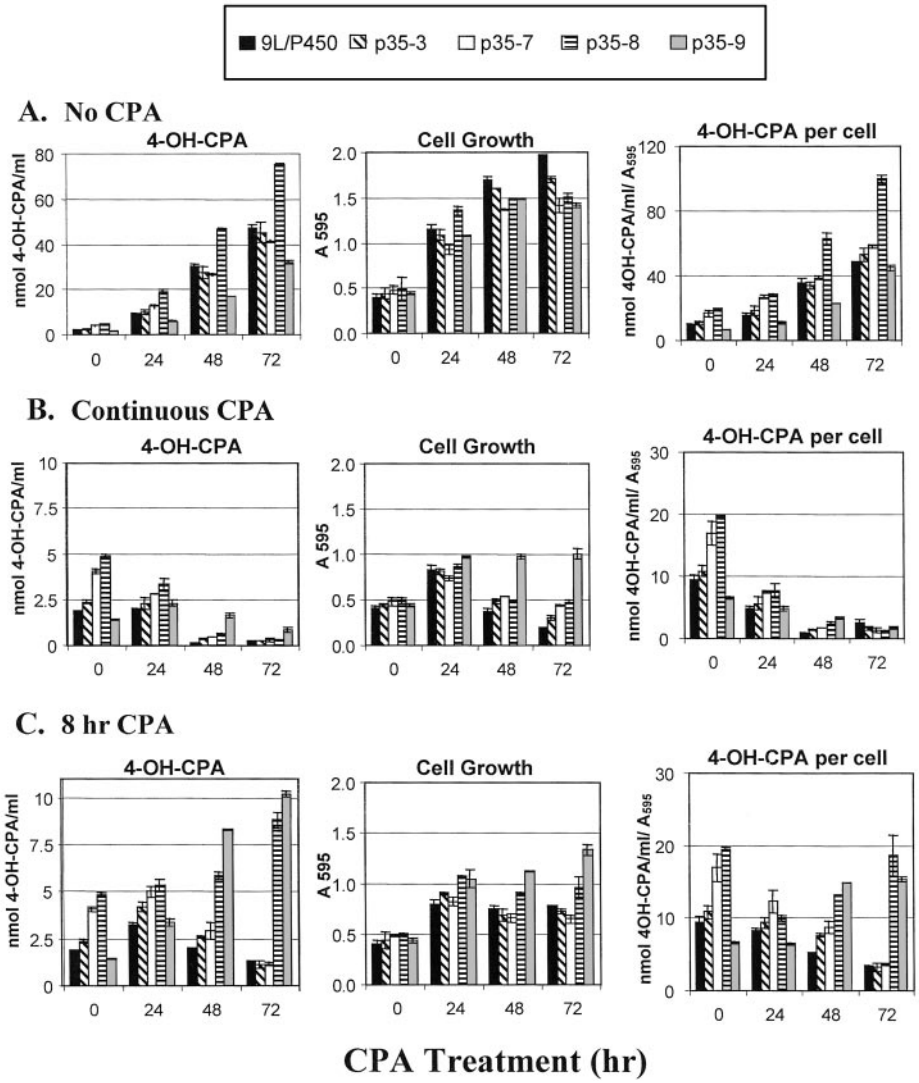

Fig. 5. Cells that express high levels of p35 retain high levels of CPA 4-hydroxylase activity after 8 h of CPA treatment. 9L/P450 cells and four 9L/P450/p35 clonal cell lines (p35–3, p35–7, p35–8, and p35–9) were untreated (*A*), were treated with 1 mM CPA continuously (*B*), or were treated with 1 mM CPA for 8 h and then cultured in drug-free medium for the duration of the experiment (*C*). At the times indicated each cell line was assayed for: cellular CPA 4-hydroxylase activity, as described in Fig. 1 (*left set of graphs in each panel*); relative cell growth rates determined by crystal violet staining (A_{595} values, *middle set of graphs*), and 4-OH-CPA production per cell protein, calculated as described in Fig. 3 (*right set of graphs*). Data shown are mean ± half the range values ($n = 2$ samples) based on a representative experiment; *bars*, ±SD.

activity of the tumor cells, measured 72 h after an initial 8-h CPA treatment. The prolonged survival of the p35-transduced tumor cells, in combination with the enhanced capacity of the tumor cell for production of active CPA metabolites, resulted in an approximately 7–8-fold overall increase in the prodrug activation capacity of the culture 3 days after an initial bolus CPA treatment. This finding was observed in a pool of tumor cells infected with retrovirus-encoding p35 and was confirmed in two independent p35-expressing clonal cell lines (Fig. 5). By contrast, when 9L tumor cells that express p35 were treated with CPA in a continuous manner, little or no increase in the specific CPA 4-hydroxylase activity of the cell was observed in comparison with the parental 9L/P450 cells. Nevertheless, tumor cell survival and the overall CPA 4-hydroxylase activity of the culture were consistently higher when the 9L/P450 tumor cells coexpressed p35. These findings suggest that there may be a threshold concentration of 4-OH-CPA, above which p35 is no longer effective at prolonging P450-dependent prodrug activation. An additional possibility is that the drug-free period after 8-h CPA treatment allows for partial recovery from drug-induced DNA damage, which may be more effective in cells that express p35.

In current clinical practice, CPA may be administered either at a single high dose or as fractionated doses given over a short period of time, based on the concept of maximum tolerable dose. Subsequent CPA treatments are typically given 2–4 weeks later, after the patient has recovered from systemic toxicity associated with the initial chemotherapy. In a second clinical regimen, smaller doses of CPA are given daily over a longer period of time (51). A third treatment regimen, which has been used in patients with advanced malignancies and is made possible by the use of autologous bone marrow transplantation, involves the administration of high doses of CPA, typically as a continuous infusion over a 4-day period (44). In the present study, optimal production of active CPA metabolites by tumor cells coexpressing P450 and p35 was achieved using a schedule of bolus CPA treatment, followed by a 3-day recovery period and then retreatment. The use of a repeated CPA dosing schedule in place of a single bolus exposure or continuous drug exposure is also supported by the findings of Browder *et al.* (52) in mouse model studies, where CPA administered at a moderate dose using a schedule of drug treatment repeated every 6 days (in the absence of P450 GDEPT) was shown to have an antiangiogenic component and was far more effective at inducing tumor cell apoptosis, yielding a higher cure rate than the conventional maximum tolerable dose approach. These findings have been confirmed and extended in *in vivo* tumor model studies, where the 6-day repeat CPA schedule resulted in complete or near complete regression of large tumors that express P450 2B6 in combination with P450 reductase (36).

Numerous studies report the impact of antiapoptotic factors, such as Bcl-2 and Survivin, and their role in tumorigenesis and chemoresis-

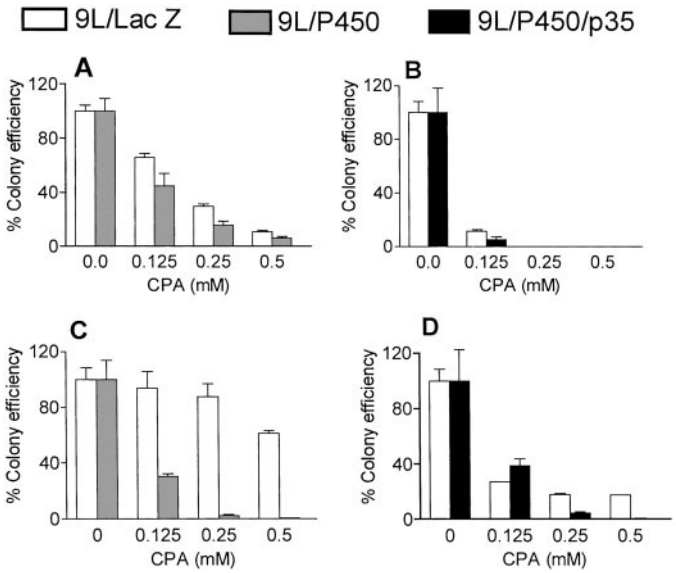

Fig. 6. Enhanced bystander cytotoxicity of 9L/P450/p35 tumor cells. The cytotoxicity conferred by 9L/P450 and 9L/P450/p35–9 cells on 9L/LacZ bystander cells was assayed using two protocols, as detailed in "Materials and Methods." Cells were cultured such that the 9L/LacZ cells were exposed to the second of two 8-h CPA treatments given to the P450-containing 9L cells. After this drug treatment cell survival was determined for each of the three cell lines (9L/P450, 9L/P450/p35–9, and 9L/LacZ) using a colony formation assay. In the experiment shown in *A* and *B* (protocol 1) the 9L/LacZ bystander cells were plated on top of the 9L/P450 or 9L/P450/p35–9 cells, enabling the two cell populations to be in direct cell-cell contact. In the experiment shown in *C* and *D* (protocol 2) the P450-containing cells were plated on cell culture inserts such that the cocultured cell lines shared the same medium but did not come in direct contact with each other. *A* and *C* present the relative colony formation activity of the 9L/LacZ cells cocultured with 9L/P450 cells, whereas *B* and *D* present the relative colony formation activity of the 9L/LacZ cells cocultured with 9L/P450/p35–9 cells. Colony formation in the absence of drug treatment was set = 100% for each sample. *Error bars* represent mean ± SD for $n = 4$ replicates.

tance (31, 53, 54). The present proposal to deliver to tumor cells an antiapoptotic factor, such as p35, in the context of a P450-based prodrug activation gene therapy may therefore seem not only counterintuitive but also potentially dangerous. Of note, therapeutic expression of antiapoptotic factors, such as p35, has been suggested for the treatment of neurodegenerative disorders such as Alzheimer's or Parkinson's disease to maintain neuronal viability (55, 56). The success of the novel approach exemplified in the present study, in which expression of an antiapoptotic factor is coupled to a suicide gene therapy, is based on the finding that the antiapoptotic factor prolongs but ultimately does not block tumor cell death. Even in tumor cells where P450 prodrug activation was submaximal and where p35 was expressed at a high level, two 8-h CPA treatments spaced 3 days apart were found to be sufficient to effect complete tumor cell death, as exemplified with the clonal tumor cell line 9L/P450/p35–9. Importantly, the presence of p35 did not result in an increase in tumor cell survival in long-term clonogenic assays. Thus, the damage induced by P450-activated CPA is sufficient to kill tumor cells, despite the presence of a strong caspase inhibitor.

Given the imprecise nature of gene delivery, there may be concern that a gene therapy vector designed to deliver p35 in combination with P450 could lead to the expression of p35 without P450 in some cells within the tumor cell population. This safety concern can be addressed by coexpression of P450 and p35 as a single transcript using an internal ribosome entry sequence or by placing the *p35* gene under the control of an inducible promoter. Furthermore, the anticipated immunogenicity of p35, a baculovirus-encoded protein, may help to ensure that all of the p35-expressing tumor cells are ultimately eliminated, thereby minimizing the risk of introducing p35. In an alternative approach, p35 may be incorporated with P450 into a cell-based therapeutic vector, such that the antiapoptotic gene is never actually introduced into tumor cells. For example, one P450 GDEPT approach, which has progressed through early Phase I/Phase II clinical trials, uses a cellulose sulfate-encapsulated cellular vector that is engineered to express P450 and can be used to enhance P450 prodrug activity within pancreatic tumors (57, 58). The use of encapsulated cells that have been genetically modified to express both a P450-prodrug activation enzyme and a caspase inhibitor such as p35 would help prolong prodrug activation within the tumor milieu while avoiding the risks associated with direct gene therapeutic intervention in the target tumor cell. Encapsulated cells offer the added advantage of circumventing immune responses, which can be significant in the case of viral vectors, such as adenoviral vectors, and may occur with p35, as noted above. Additional investigation into the immunogenic potential of p35 is warranted. If p35 does, in fact, trigger a strong and early immune response that compromises its ability to prolong prodrug activation, then a nonimmunogenic human IAP, such as Survivin (Fig. 2), could be used in its place.

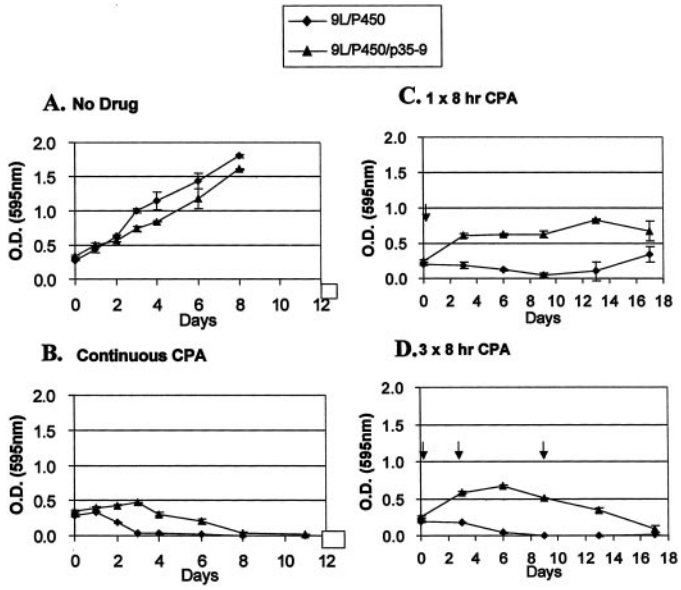

Fig. 7. Time course for killing of 9L/P450 and 9L/P450/p35 cells by CPA. 9L/P450 and 9L/P450/p35–9 cells were untreated (*A*), were treated with 1 mM CPA continuously (*B*), or were given a single 8-h treatment (*C*) or three 8-h treatments (*D*) with 1 mM CPA. Cells were then cultured for up to 17 days after the initial drug treatment. *Downward arrows* in *C* and *D* indicate the times at which each 8-h CPA treatment was initiated. Cells remaining on the plates at each indicated time point were stained with crystal violet. Data shown are mean ± half the range for A_{595} values; $n = 2$ samples at each time point; *bars*, ±SD.

Table 1 *Effect of CPA treatment on colony formation activity of 9L/P450 and 9L/P450/p35-9 cells*

Cells plated in 12-well plates at 1.5×10^5 cells/well/1.5 ml culture medium were untreated or were treated with 1 mM CPA for a single 8-h CPA treatment (day 0) or for two 8-h CPA treatment, on day 0 and day 3, respectively. Six days after the initial CPA treatment the cells remaining on each plate were counted and replated in duplicate as described in "Materials and Methods." Eleven days after replating, the number of colonies (≥50 cells each) that had formed under each treatment condition was counted. Data shown represent mean ± SD for $n = 3$ and are based on cells plated at densities of 100 (untreated) or 1,000, 5,000, or 10,000 cells per well of a six-well dish (CPA-treated samples). Where indicated ([a]), colony formation activity was significantly different between 9L/P450 and 9L/P450/p35-9 cells, as determined by two-tailed, two-sample equal variance *t* test ($P < 0.02$). Relative colony formation activities are shown in parenthesis.

Treatment	Colonies per 10,000 cells	
	9L/P450	9L/P450/p35-9
No Drug	6950 ± 150 (100 ± 2.1)	5250 ± 250 (100 ± 4.7)
1 × 8 h CPA	77 ± 35 (1.1 ± 0.5)	150 ± 49 (2.9 ± 0.9)[a]
2 × 8 h CPA	0 ± 0	0 ± 0

[a] $P < 0.02$.

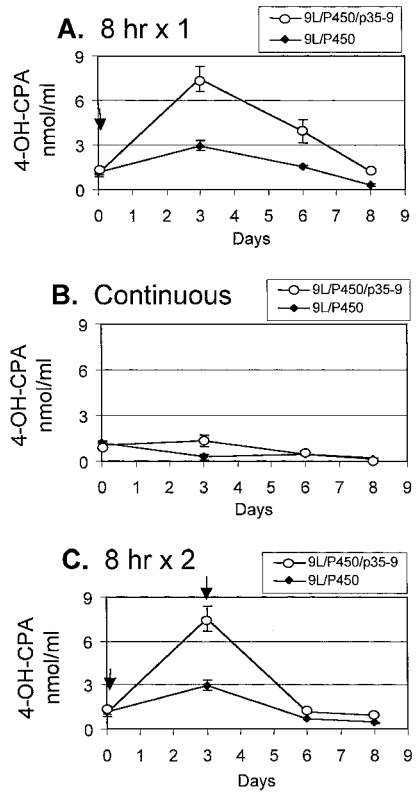

Fig. 8. 9L/P450/p35–9 cells loose CPA 4-hydroxylase activity after two 8-h CPA treatments. CPA 4-hydroxylase activity of 9L/P450 and 9L/P450/p35–9 cells was assayed as described in Fig. 1 in cultures treated with 1 mM CPA given as a single 8-h exposure (*A*), continuously (*B*), or for two 8-h exposures spaced 3 days apart (*C*). *Downward arrows* indicate the times when the 8-h CPA treatments were applied. Data shown are mean ± half the range A_{595} values for $n = 2$ samples at each time point; *bars*, ±SD.

The present finding that P450-expressing tumor cells treated with CPA ultimately die, even in the presence of high levels of the potent caspase inhibitor p35, demonstrates that caspase activation is not a prerequisite for CPA-induced cell death. Conversely, the fact that the antiapoptotic factor Bcl-2 does increase the clonogenic survival of tumor cells exposed to activated CPA (9) indicates that mitochondria play a pivotal role in regulating CPA-induced apoptosis. The ability of Bcl-2 but not p35 to block CPA-induced cell death may hinge on the fact that Bcl-2 but not p35 blocks mitochondrial transition (31). Multiple proapoptotic factors are released into the cytosol during mitochondrial transition, including caspases (18), cytochrome C (16, 19), AIF (59, 60), and Smac/Diablo (61, 62), all of which can contribute to the execution of apoptosis. Our finding that the long-term survival of CPA-treated 9L/P450 tumor cells is not increased by p35 expression additionally suggests that CPA-induced mitochondrial transition and the ensuing apoptotic responses are not blocked by IAPs. Moreover, given its ability to prolong tumor cell death and increase bystander activity without ultimately increasing tumor cell survival, p35 is well suited for incorporation into P450-based gene therapies. By contrast, whereas Bcl-2 overexpression may also allow for prolonged CPA 4-hydroxylase activity, the enhanced tumor cell clonogenic survival and CPA chemoresistance that accompany Bcl-2 expression (9) make Bcl-2 less well suited for this application.

The release of the proapoptotic protein Smac/Diablo into the cytosol during mitochondrial transition is of particular interest insofar as a primary function of this protein is to bind to and neutralize the inhibitory activities of several IAPs, including hIAP1, hIAP2, XIAP, and Survivin (61–63). The possibility of mitochondrial release of Smac/Diablo in tumor cells exposed to 4-OH-CPA, in combination with the comparatively weak intrinsic inhibitory constant of the human IAPs, hIAP1, hIAP2, and Survivin, in comparison with that of p35 (31), could contribute to the incomplete caspase inhibition that was observed in 9L/P450 cell lines that stably express these factors. Additionally, the release of Smac/Diablo could explain why high levels of p35 are needed to prolong CPA-induced tumor cell death. AIF is also released from mitochondria and may contribute to CPA-induced cell death in the presence of the caspase inhibitor p35 (59), insofar as AIF release can stimulate caspase-independent programmed cell death. AIF is sequestered in the mitochondrial intermembrane space, and when released to the cytosol in response to apoptotic stimulation, translocates to the nucleus where it causes large-scale chromatin fragmentation (60). This process is not blocked by the general caspase inhibitor z-VAD-fmk, although, the release of AIF from mitochondria is regulated by Bcl-2 (18).

Baculovirus has evolved the ability to express p35, enabling this insect cell virus to suppress the host cell death response to viral infection and thereby maximize virus production. The concept of using p35 to enhance P450 GDEPT, exemplified for the prodrug CPA in the present study, is expected to be directly applicable to P450 GDEPT using other clinically established P450 prodrugs, some 10–12 of which are known (6). Additional antiapoptotic approaches, including expression of mammalian IAPs, Bcl-2 family members with antiapoptotic activity, antisense or small inhibitory RNA targeting caspases, and expression of factors that regulate receptor-mediated cell death, such as the CD95 inhibitor FLIP or Trail decoy receptors, are alternative approaches that may be coupled with cytotoxic gene therapies that benefit from an enhanced bystander effect. Other prodrug activation therapies that might be improved in this manner include those that use herpes simplex virus thymidine kinase, cytosine deaminase, or other suicide genes (49, 64). Factors that determine the extent of enhancement that can be achieved include the mechanism of cell death induced by the activated prodrug, the extent to which the antiapoptotic factor prolongs cell death, and the extent to which bystander killing is enhanced by expression of the antiapoptotic factor. The ability of IAPs to prolong prodrug-induced cell death and enhance the net production of activated drug metabolites in a manner that does not increase tumor cell survival may thus serve as a general way to enhance a broad range of GDEPT-based cancer treatments.

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